PHYSIOLOGY OF INSECT DIAPAUSE. XIII. DNA SYNTHESIS DURING THE METAMORPHOSIS OF THE CECROPIA SILKWORM

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Ten micrograms of ecdysone injected into a brainless, diapausing Cecropia pupa results in the termination of diapause within 36 hours (Williams, 1954). The same result is seen when the injection is into an isolated pupal abdomen. Without the injection, neither the brainless pupa nor the isolated abdomen would be able to initiate adult development. The abdomen will not develop because of the absence of prothoracic glands. The brainless pupa will not develop because the prothoracic glands, though present, are unable to secrete ecdysone in the absence of the tropic stimulus of brain hormone. Yet almost overnight, the injection of a minute amount of ecdysone terminates the "permanent" diapause and swiftly mobilizes the dormant cells and tissues in a complex and coordinated developmental response.

Manifestly, this experiment is a caricature of what goes on within diapausing pupae in the spring of the year. The brain hormone is secreted and drives the prothoracic glands; then over a period of a week or two, the pupa gives *itself* the ecdysone injection.

The termination of diapause, whether naturally achieved or experimentally induced, focuses attention on endocrinological events at the cellular level. How does ecdysone, after months of developmental standstill, restore to the diapausing cells their capacity to grow?

The present approach to the problem has been motivated by our previous unpublished cytological studies of diapausing and post-diapausing tissues. In preparation of diapausing tissues one cannot fail to be impressed by the absence of any signs of mitotic activity—a generalization to which the hemocytes and spermatogonia seem to constitute the only exceptions. By contrast, the action of ecdysone and the initiation of adult development are accompanied in the vast majority of tissues by what is little short of a mitotic explosion.

Notwithstanding this fact, there are cogent reasons for disbelieving that ecdysone acts by stimulating cell division. For example, the response of most larval tissues to ecdysone includes growth by a combination of mitosis and cell enlargement, the latter being accompanied by chromosome replication. Indeed, it will be recalled that in the higher Diptera and Hymenoptera, the growth of larval tissues seems to be solely by cell enlargement, including chromosomal replication. So, in cytological terms, it is chromosomal replication rather than cell division which seems to distinguish between growing and diapausing cells.

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In the present investigation we have sought to test this hypothesis by means of an autoradiographic study of DNA synthesis in diapausing and non-diapausing Cecropia and Cynthia silkworms.

Methods

1. Experimental animals

The experiments were performed on male larvae and pupae of *Hyalophora cecropia* (L.) and male pupae of *Samia cynthia* (Drury). The Cecropia were reared on wild-cherry trees; the Cynthia were obtained from dealers.

Large injuries were made on diapausing Cynthia pupae as described by Harvey and Williams (1961).

2. Injections

Fifth instar larvae were anesthetized with carbon dioxide and a dorsal tubercle of the sixth abdominal segment was excised with scissors. The injection of thymidine was made through the tubercle from a 0.25-ml. hypodermic syringe *via* a 27-gauge needle, and the opening immediately sealed with melted wax. Each larva received 4 or 5 μc. of tritiated thymidine per gram live weight. The caterpillars were kept at room temperature for 6 to 27 hours before fixation.

Pupae were injected, without anesthesia, in the mesothoracic dorsum just lateral to the mid-line, and the puncture wound sealed promptly with melted wax.

Table I

Summary of animals studied

Animal #	Stage	H^3-thymidine per g. body wt.		Time from injection to fixation (hours)
		μc.	mμmoles	
L1	Late 5th instar (4.1 g.)	5	3	6
L2	Late 5th instar (4.4 g.)	5	3	18
L3	Late 5th instar (4.7 g.)	4	2	27
L4	Spinning outer cocoon	4	2	19
L5	Early prepupa (finished inner cocoon; eye epidermis had retracted)	4	2	19
P1	6–8 days after larval-pupal molt	4	2	19
P2	7 days after larval-pupal molt	6	2	18
P3	14 days after larval-pupal molt	6	2	18
P4	Diapause: 2–3 months after pupation	7	8	19
P5	Diapause: 5 months after pupation (Q_{O_2}:15.5)*	4	2	18
P6	Diapause: 5 months after pupation (Q_{O_2}:9.6)*	4	2	18
P7	Diapause (Q_{O_2}:9.1)* placed at 6° C. for 10 weeks; fixed at 6° C.	6	7	1680
P8	Diapause (Q_{O_2}:10.5)* placed at 6° C. for 10 weeks; fixed at 6° C.	6	4	1680
P9	Chilled 10 weeks at 6° C. Injected after return to 25° C.	6	2	6
P10	Chilled 10 weeks at 6° C. At 25° C. for 7 days (Q_{O_2}:57)*	6	2	27
P11	Chilled 10 weeks at 6° C. At 25° C. for 11 days (Q_{O_2}:55)*	6	2	27
A1	Reached day 0 of adult development at 6° C.; injected when removed to 25° C.	4	2	19
Cynthia				
P12	Diapause: 2–3 months after pupation	10	11	27
P13	Diapause: 6–7 months after pupation	10	11	2880
A2	Day 2 of adult development	4	4	18
A3	Day 2 or 3 of adult development	6	7	27
A4	Day 3 of adult development	10	11	18
I1	Three days after injury	4	1	18
I2	Six days after injury	4	1	18
I3	Six days after injury	4	1	18
I4	Nine days after injury	4	1	18

* Oxygen consumption measured in Warburg manometers, usually for a five-hour period; expressed as μl. oxygen consumed per gram live weight per hour.

The volume of each injection was 0.01 to 0.02 ml., and each pupa received 4 to 10 μc. of the thymidine. The pupae were stored at 25° C. or at 6° C. until fixed. Table 1 summarizes the status and treatment of the 26 animals that were studied.

3. Thymidine

The tritium-labeled thymidine was from three batches: New England Nuclear Corp. (Boston, Mass.), with a specific activity of 890 millicuries per millimole; Schwarz Laboratories, Inc. (Mt. Vernon, N. Y.), with a specific activity of 1600 mc./m*M*; and Schwarz, with a specific activity of 3000 mc./m*M*.

4. Fixation and embedding

In the early experiments, the wings and testes of the injected individuals were dissected from unanesthetized animals and fixed in one of several standard fixatives: Bouin, Helly, Carnoy (6:3:1), Flemming (0.4 gram osmic acid, 0.72 gram chromic acid in 100 ml. water), or 95% ethanol-glacial acetic acid (3:1). One of the paired structures was fixed in each of two solutions.

In later experiments, entire animals were fixed in 10% aqueous acrolein at room temperature for two hours, the cuticle being sliced to facilitate entrance of the fixative. As the tissue hardened, the initial incisions were extended to transect the animals. They were post-fixed in Methyl Formcel (Celanese Corp., Framingham, Mass.) for 12 to 24 hours and dehydrated in methanol. Two animals (P2, P3) were fixed in 10% acrolein at 0° C. and dehydrated in ethylene glycol monomethyl ether and methanol, as described by Feder (1960).

The fixed material was embedded in paraffin wax (Fisher Tissuemat, M.P. 58–60° C.) or in polyester wax (Sidman *et al.*, 1961). The sections were routinely cut at six micra. Since about 90% of the tritium radiation is absorbed in 1.2 μ of tissue (Fitzgerald *et al.*, 1951), these sections were "infinitely thick" with respect to tritium disintegrations.

5. Preparation of radioautograms

Radioautograms were prepared as described by Messier and Leblond (1957). The slides were coated with Kodak Nuclear Emulsion, Type NTB 3; the latter had a thickness of approximately five micra.

After exposure times of 1 to 6 weeks at −20° C., the radioautograms were developed by 1½ minutes' immersion in Kodak developer D-72, followed by 10 minutes in Eastman formula F-5 acid fixing bath. The slides were rinsed at least 45 minutes in 4 or 5 changes of tap water. They were then taken directly to the staining solution. The stain routinely employed in this study was 1% aqueous toluidine blue, but some slides were stained by the Feulgen procedure (Stowell, 1945) before coating. The sections were finally dehydrated, cleared, and mounted in damar.

The dose of isotope was always high enough to give unequivocal radioautograms (4 to 40 grains per nucleus) after seven days' exposure. In each case some sections were exposed for 14 and 28 days, to check for very low levels of labeling. The cells were scored either as "labeled" or "unlabeled."

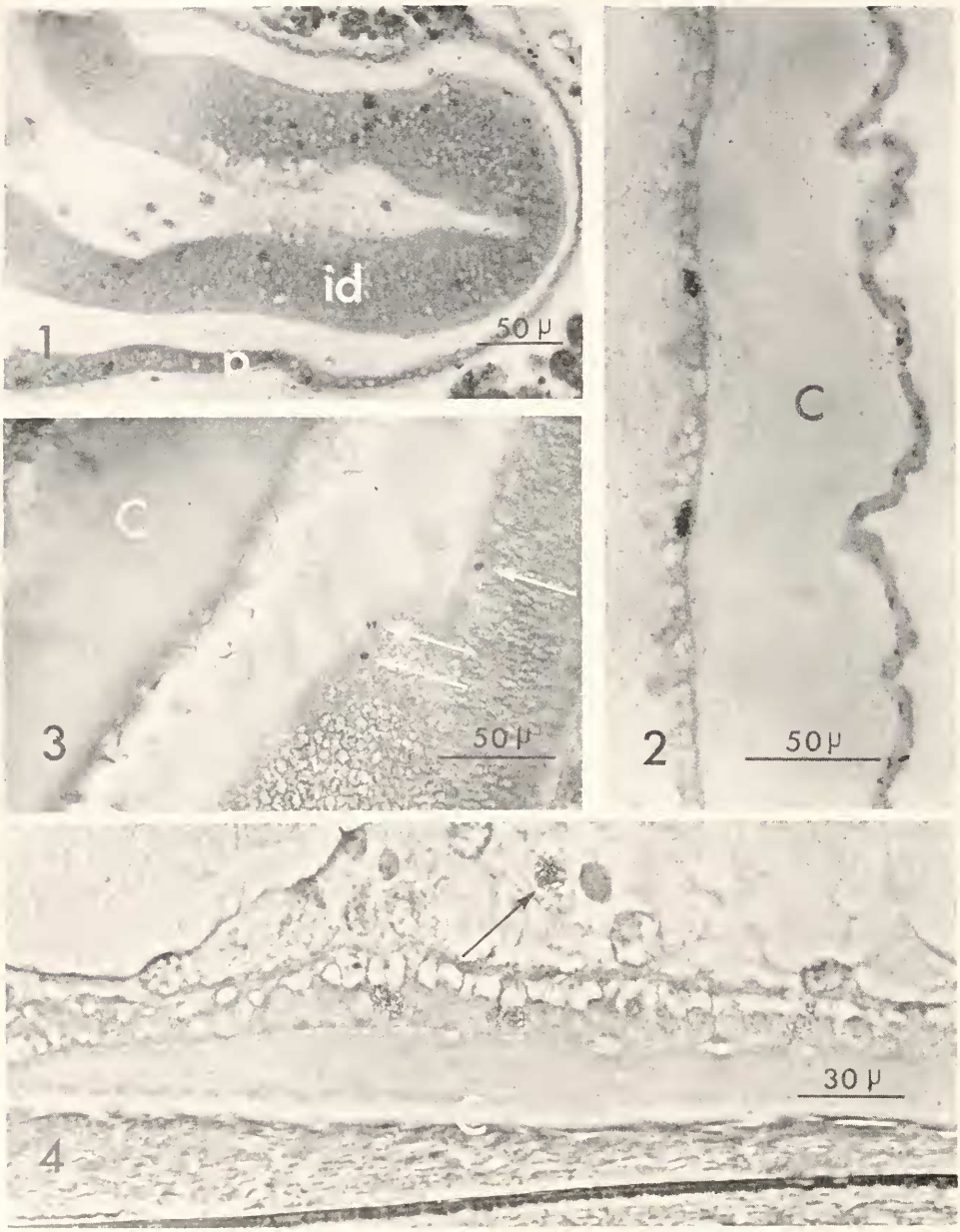

FIGURE 1. Radioautogram of a section of wing disc from a late fifth instar Cecropia. Nuclei are unstained. id, imaginal disc; p, peripodal membrane.

FIGURE 2. Radioautogram of a section of wing of a Cecropia pupa one week after pupal ecdysis. Presence of radioactive nuclei indicates DNA synthesis by epidermal cells at this time. Note the accumulations of silver grains in the cuticle (C), probably due to reducing compounds such as polyphenols.

6. Specificity of thymidine as a DNA precursor

In sections of tissues fixed in Bouin's or Helly's fluids, the extraction of RNA and unincorporated precursors of the nucleic acids was accomplished by hydrolysis with 10% perchloric acid at 20°C. for two hours (Woods and Taylor, 1959; Atkinson, 1952).

DNA was selectively extracted from sections fixed in 95% ethanol:glycol acetic acid (3:1) by means of crystalline deoxyribonuclease (Nutritional Biochemical Corp., Cleveland, Ohio). The enzyme was prepared in Gomori's Tris-maleic acid buffer (Gomori, 1952), adjusted to 0.02 *M* with respect to $MgSO_4$ and to pH 5.95 with 1 N NaOH (McDonald, 1955). The concentration of the solution was 5 mg. of enzyme in 100 ml. of buffer. Slides were incubated for 18 hours at 37° C. (Amano *et al.*, 1959). Control slides were incubated with buffer in the absence of enzyme. After incubation, both the DNase-digested and the control slides were rinsed in distilled water, hydrolyzed 8 minutes in 1 N HCl at 60° C., and stained with Schiff reagent. The slides were coated with emulsion by the procedure described above.

Hydrolysis in 10% perchloric acid did not remove the radioactivity localized over the nuclei; this indicates that the label was in polymerized DNA. Slides incubated in DNase showed no Feulgen staining and gave no radioautogram. The control slides, consisting of sections adjacent to those digested, retained their nuclear-localized radioactivity and the capacity to stain with Schiff reagent.

Experimental Results

1. The wing anlagen

In larval Lepidoptera the anlagen of the future wings consist of two pairs of imaginal discs situated on each side of the meso- and metathorax just internal to the epidermis. Each disc takes the form of a flattened pouch of pseudostratified epithelium, which is invaginated into and surrounded by a thinner envelope of epithelium, the latter forming the so-called peripodal sac. During successive stages of larval life the wing anlagen undergo a steady increase in size; their dry mass, in fact, shows the same relative growth rate as does the larva itself (Williams, unpublished measurements). At the onset of the prepupal period—a stage recognizable by the retraction of the pigment granules of the ocelli (Kühn and Piepho, 1936)—the wing discs undergo a sudden spurt in growth and are drawn to the outside of the body to lie beneath the loose cuticle of the final larval instar. The shedding of this cuticle at the time of the pupal ecdysis reveals the prominent wing-pads of the pupa itself. Each consists of a flattened sac of epithelium. Unlike the imaginal discs, the surfaces of pupal wings are enticularized; this is particularly true of the outer exposed surface of the forewings.

Figure 3. Epithelium in the head region of a Cecropia pupa one week after the pupal ecdysis. Arrows indicate dividing epithelial cells. This is a stained section and not a radioautogram. C, cuticle.

Figure 4. Radioautogram of a section of a wing of a Cecropia pupa shortly before the visible initiation of development. In this phase contrast image, some of the silver grains appear as bright spots. The epidermis still adheres tightly to the cuticle (C). Arrow indicates blood cell which shows incorporation.

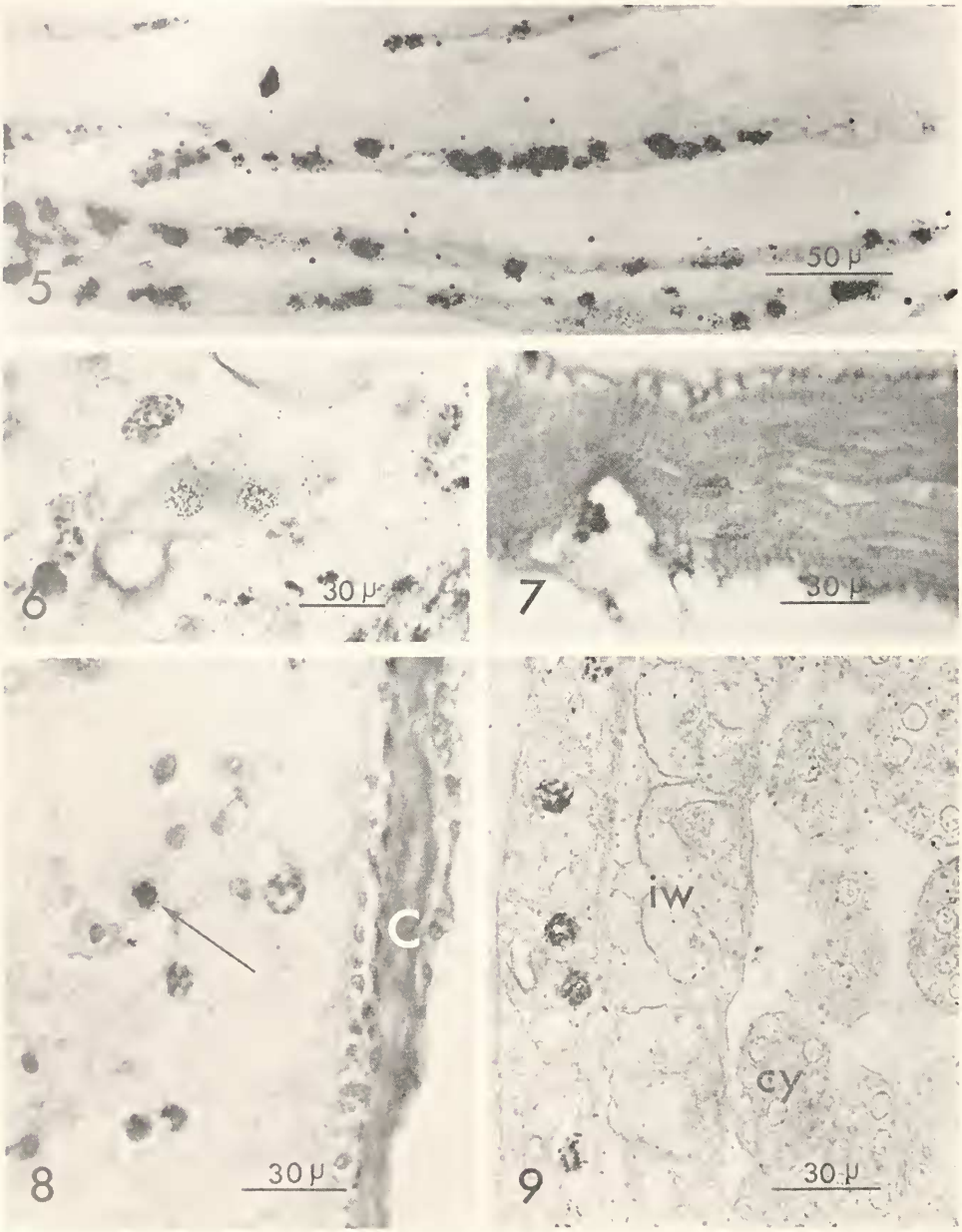

FIGURE 5. Radioautogram of a section of wing of a Cecropia pupa at "day 1" of adult development. Nearly half of the epidermal cells have incorporated tritiated thymidine. The slide was exposed for four weeks; hence the very heavy deposits of silver.

FIGURE 6. Radioautogram of a section of a Cecropia pupa before the visible initiation of adult development. The nuclei of two tracheoblasts have incorporated tritiated thymidine.

After the sclerotization of their cuticle, the pupal wings undergo no obvious change during the true pupal period. In non-diapausing insects this stage lasts only a few days before adult development begins. However, in diapausing pupae, such as that of the Cecropia silkworm, the pupal stage persists for about 8 months. Then, with the initiation of adult development, the epidermis of the wings retracts from the overlying pupal cuticle and enters at once into a swift and complicated program of differential cell divisions which has been described in such impressive detail by Henke and his students (for summary, see Kühn, 1955).

In the present study, the incorporation of tritiated thymidine into the DNA of the nuclei of the cells of the wing anlagen was studied in a series of 17 Cecropia ranging from the fifth larval instar to an early stage in adult development.

In line with the rapid growth of the wing during the larval and prepupal periods, incorporation of label (Fig. 1), as well as mitotic figures, was detected in all of a series of five fifth-instar larvae described in Table 1. This was true of the disc itself as well as the surrounding peripodal sac. In all cases the labeled cells and the mitotic divisions appeared to be in localized clusters scattered through the wing tissues. Mitotic activity was highest in the discs of a mature larva which was in process of spinning its cocoon. Incorporation of label was maximal in the early prepupal stage where about 17% of cells showed DNA synthesis within a period of 19 hours.

A surprising finding was a continuation of DNA synthesis in the wings of pupae seven days after the pupal ecdysis (see Fig. 2). Synthesis continued at this stage despite the apparent absence of mitotic divisions. Two weeks after the pupal ecdysis, all incorporation of label ceased. Four diapausing Cecropia pupae (P3, P4, P5, and P6) showed no trace of DNA synthesis after storage at 25° C. for as long as five months.

Exposure to low temperatures is known to potentiate the initiation of adult development by restoring the brain's endocrine activity. Thus, after ten weeks at 6° C., Cecropia pupae initiate development within several weeks after their return to 25° C. (Williams, 1956). DNA synthesis at the low temperature was studied in two pupae (P7, P8) which were injected with tritiated thymidine and immediately placed at 6° C. Ten weeks later, the animals were sacrificed without rewarming them. The wing epithelia showed no incorporation of label during this long exposure. In a third pupa (P9) the injection of label was postponed until the end of the ten weeks of chilling. After six hours at 25° C., the wings of the pupa likewise showed no DNA synthesis.

Of particular interest are two further pupae of this same series. These were subjected to ten weeks of chilling but the injection of label was further postponed

FIGURE 7. Radioautogram of a section of a Cecropia pupa before the visible initiation of adult development. Incorporation of triated thymidine is evident in the nuclei of an intersegmental muscle of the abdomen.

FIGURE 8. Radioautogram of a section of a wing of a Cynthia pupa injected with tritiated thymidine six days after an integumental injury. The epidermal cells show no incorporation of label; one blood cell (arrow) in this field shows label.

FIGURE 9. Radioautogram of a section of a testis of a Cynthia pupa at "day 2–3" of adult development. Five nuclei of the outer wall of the testis are radioactive. None of the spermatocytes are labeled. iw, inner wall of testis; cy, spermatocyte cyst.

after their return to 25° C. P10 was stored at 25° C. for seven days, at which time its Q_{O_2} had increased to 57 μl. per gram live weight per hour. P11 was stored at 25° C. for eleven days and showed a Q_{O_2} of 55. Both animals were then exposed to tritiated thymidine for 27 hours at 25° C. Despite the fact that neither pupa showed any obvious trace of the initiation of adult development, approximately 14% of the cells of the wings were labeled (Fig. 4).

After very prolonged storage at 6° C., Cecropia pupae show a retraction of facial and wing epidermis and other signs indicating the initiation of adult development at the low temperature (Williams, 1956). One pupa of this type was studied (A1). This animal was injected with isotope and placed at 25° C. for 19 hours. The wings showed numerous mitotic divisions and a high percentage of labeled cells (up to 47% in certain sections). A radioautographic section of a wing of this animal is shown in Figure 5.

The above-mentioned results were confirmed in a less extensive study of the Cynthia silkworm. Here, no incorporation of label was found in the wing epithelium of two diapausing pupae stored at 25° C. (P12, P13). By contrast, three animals (A2, A3, A4), ranging from "day 0" to "day 3" of adult development at the time of injection, showed frequent mitotic figures and substantial incorporation of label.

2. The testes

The male gonads are paired, kidney-shaped organs lying dorsally on each side of the heart in the fifth and sixth abdominal segments. Each testis consists of four compartments surrounded and bound into one complex by a testicular capsule. The gross morphology of the testis, unlike that of the ovary, does not change during metamorphosis.

The capsule of the testis consists of two distinct layers (Fig. 9), the inner of which is continuous with the three septa which subdivide the testis into four compartments. The cells of the inner layer seem to function as a storage tissue during the pupal period. The entire capsule is heavily laced with trachea and tracheoles which supply the cells therein and pass into the lumen to supply the germinal cells.

Primordial germ cells appear in the cavities of the testis at an early embryonic stage. They become localized at the expanded blind end of each of the four compartments and, by repeated mitotic divisions, give rise to spermatogonia throughout larval life. The spermatogonia become isolated in spherical clusters of 16 to 24 cells and enveloped by follicle cells (Cook, 1910). The latter are mesothelial cells which envelop the spermatogonia as a simple squamous sheath. The whole complex forms the so-called "germinal cyst." By the late fifth instar, the four chambers of each testis are packed with these cysts. Approximately one-third contain spermatogonia, one-third contain spermatogonia in synapsis, and one-third contain primary spermatocytes. All types of pre-meiotic cysts persist in the testes in varying proportions until the pupa terminates diapause and initiates adult development. At this time the primary spermatocytes undergo meiosis and proceed with spermiogenesis. During the 21-day period of adult development all of the germinal cysts mature into spermatozoa.

During larval life the testes grow at the same relative rate as the larva itself. But, unlike the wing anlagen, which undergo a spurt of growth during the prepupal

period, the testes respond to pupation as if it were just another larval molt (Williams, unpublished measurements).

In the present study we found that tritiated thymidine was incorporated by the spermatogonia, but not by primary spermatocytes or any subsequent stage. The spermatogonia incorporated the label at each period in the life-cycle—even during pupal diapause. In like manner, mitotic figures were evident in the spermatogonia in each animal which was examined, including the diapausing pupa. No incorporation of label was observed in the follicle cells at any stage from mature larva to early adult development.

If attention is directed to the cells which comprise the capsule and walls of the testis, here, again, at least an occasional cell was found to incorporate label at all stages including the diapausing pupa. The synthesis of DNA was particularly prominent in the outer layer of the capsule in the early prepupa (L5) and at the outset of adult development (A1, A3) (Fig. 9).

In summary, we find that the spermatogonia show a contrasting behavior to that of the wing epithelium, in that the synthesis of DNA is not completely shut off during the pupal diapause. The cells of the testicular wall show a maximum of DNA synthesis at the same periods in which the wing epithelium synthesizes DNA; in addition, a few cells incorporate label during diapause.

3. Blood cells

These cells incorporated thymidine at every stage surveyed. Labeled blood cells were observed in the lumen of the wings and legs, in the head, in the abdomen—in short, throughout the entire animal. The percentage of labeled cells varied from 1% to 10% without any discernible relation to developmental stage. Mitotic divisions were seen in a prepupa, a diapausing pupa, and in a pupa just prior to initiation of adult development.

4. Midgut

The midgut of the pupa consists of a columnar epithelium with a brush border, subtended by layers of circular and longitudinal muscles. The columnar cells are derived from crypts of regenerative cells at the base of the epithelium (Wigglesworth, 1950). As might be expected, among the epithelial cells only the regenerative ("crypt") cells showed any incorporation of isotope. Labeling of cells was not observed in an animal one week after the pupal molt; but two weeks after pupation, and in diapausing animals stored at 25° C. or at 6° C., a few of these cells were labeled. The largest number of radioactive regenerative cells was found in an animal chilled for ten weeks and returned to room temperature for six hours (P9). During the early stages of adult development (P10, P11), the crypt cells had increased in number over the diapause condition, but only rarely showed any evidence of DNA synthesis.

The muscular layer of the midgut is interlaced with trachea and highly infiltrated by blood cells. This region showed a few cells incorporating at each stage, but the radioactivity observed here can most probably be attributed to blood cells.

5. Other tissues

The epidermal cells of the pupal head and antennae showed mitotic divisions and incorporation of label one week after the pupal ecdysis (Fig. 3). However, within the following week these activities disappeared (P3) and remained absent throughout the pupal diapause (P4, P5, P6, P7, P8, P12, P13).

Synthesis of DNA was also detected in the thoracic myoblasts and cells of the central nervous system one week after the pupal ecdysis (P2). Within the following week the myoblasts showed no further synthesis, whereas an occasional cell of the central nervous system continued to incorporate label. All these activities came to a halt after the onset of pupal diapause, *i.e.*, approximately two weeks after the pupal molt.

Prior to the retraction of the pupal wing epidermis, signaling what has been called the "zero day of adult development," the resumption of DNA synthesis was already evident (P10, P11) in the cells of the central nervous system, the tracheoblasts (Fig. 6), the myoblasts of the abdominal segments, and the nuclei of the intersegmental muscles of the pupal abdomen (Fig. 7). The cells of the fat body did not resume DNA synthesis until after the zero day of adult development (A1).

The cells of the Malpighian tubules showed no incorporation of label at any stage that was examined.

6. Effects of integumentary injury to diapausing pupae

The effects of large integumentary injuries were studied on four diapausing Cynthia pupae at 25° C. The animals were injured and tritiated thymidine was injected 3, 6, and 9 days later; after 18 hours, the pupae were sacrificed and examined.

No resumption of DNA synthesis was detectable in the wing epidermis (Fig. 8), the abdominal epidermis, or the intersegmental muscles of the abdomen. However, radioactive nuclei were encountered in the thoracic myoblasts, in an occasional regenerative cell of the midgut and in a few epidermal cells in the immediate vicinity of the wound.

The most spectacular effect of injury was on the blood cells. After 18 hours of exposure to label, about 30% showed DNA synthesis. The labeled cells accumulated in largest numbers at or near the wound, but were also found throughout the animal as a whole. Mitotic figures were seen in all the injured pupae, although they were more numerous in the animal nine days after injury.

Discussion

1. The chronology of DNA synthesis

a. The pre-diapause pupa

During the first two weeks after the pupal ecdysis the rate of oxygen consumption declines to a low level which then persists during the months of pupal diapause (Schneiderman and Williams, 1953). The entire endocuticle, making up about 80% of the mature pupal cuticle (Passonneau and Williams, 1953), is secreted during this two-week period (Harvey and Williams, unpublished observations)—a

finding which documents a continuation of synthetic operations within the freshly pupated insect.

In the present study this conclusion was further reinforced by the finding that DNA synthesis and mitotic divisions continue in many tissues immediately after the pupal molt. This suggests the possibility that, in Cecropia at least, a limited progress toward differentiation of the adult may occur at this time.

b. The diapausing pupa

The onset of diapause may be dated from approximately 10 to 14 days after pupal ecdysis at 25° C. By the fourteenth day, DNA synthesis could no longer be detected in the epithelial cells of the hypodermis. This condition of arrested synthesis then persists during the months of pupal diapause, irrespective of whether the pupae are stored at 25° C. or 6° C.

The present investigation has identified two types of cells which are exceptional in that both DNA synthesis and mitosis apparently continue after the pupa is firmly established in diapause. These cells include the hemocytes and the spermatogonia. Labeled nuclei were occasionally observed among the cells of the testicular walls and the regenerative cells of the midgut epithelium, but no mitotic divisions were seen in these tissues.

c. Injured diapausing pupae

The full significance of the just-mentioned observations is not wholly clear, for the reason that the injection of tritiated thymidine constitutes an injury which in itself swiftly mobilizes many of the metabolic and synthetic activities of diapausing pupae (Shappirio, 1958, 1960; Telfer and Williams, 1960; Skinner, 1960, 1963; Laufer, 1960; Stevenson and Wyatt, 1962). Therefore, the experiments performed on diapausing Cynthia pupae subjected to large integumentary injuries are of particular interest and importance.

With few exceptions those types of cells which show no DNA synthesis in injected but otherwise uninjured pupae continued to show no synthesis when the injection was preceded by a large injury. A few thoracic myoblasts and an occasional epidermal cell in the immediate vicinity of the wound constitute the exceptions to this generalization.

Attention is now directed to those types of cells which show a low level of incorporation of injected thymidine, *e.g.*, cells of the testicular wall. Only in the case of the hemocytes did large integumentary injuries provoke any noteworthy increase in the number of cells showing DNA synthesis.

Thus, we find that injury to a diapausing pupa is a potent stimulus for DNA synthesis in the hemocytes. For all other tissues it seems, at most, to be an extremely feeble stimulus.

d. The post-diapause pupa

After ten or more weeks at 6° C., the pupal brain regains its ability to secrete the brain hormone which promotes the initiation of adult development by "turning on" the prothoracic glands. At 6° C. the secretion and translocation of brain hormone occur slowly and are complete after approximately ten months (Williams,

1956). Then, despite the low temperature, adult development progresses as far as retraction of the wing epidermis from the overlying pupal cuticle.

If the period of chilling is less prolonged, adult development begins only after the pupa has been placed at higher temperatures for one to two weeks. Approximately one week prior to the retraction of wing epidermis, the rate of oxygen consumption begins to increase.

As pointed out by Schneiderman and Williams (1953), this gradual increase in respiration indicates that the biochemical events associated with the termination of diapause are initiated at least one week prior to any morphological sign of development. The results of the present study are in full accord with this interpretation. During the week prior to the visible initiation of adult development, DNA synthesis is resumed or accelerated in the vast majority of tissues. The earliest response is seen in the regenerative cells of the midgut, followed shortly thereafter by the epidermal cells of the wings, the intersegmental muscles of the abdomen, tracheoblasts, myoblasts, and the cells of the central nervous system.

c. The developing adult

The detachment and retraction of the epidermis occur first in the region of the eye lobes and genitalia and then swiftly spread to the wings. It is clearly evident in the legs by the second day of adult development at 25° C.

Though the majority of tissues show a resumption or acceleration of DNA synthesis before the retraction of the epidermis, the fat body is exceptional in that its response is delayed until the first few days of adult development.

2. Hormones and DNA synthesis

a. Biochemical changes

DNA replication may be included in a long list of synthetic activities which subside immediately prior to the onset of pupal diapause, remain at a low level for months thereafter, and accelerate during the termination of diapause and initiation of adult development (Gilbert and Schneiderman, 1961; Harvey, 1962; Wyatt, 1959, 1962, 1963). As mentioned earlier, this same ebb and flow is seen in the overall metabolism in terms of the rate of oxygen consumption of the animal as a whole.

All these systematic alterations are of special interest because they proceed in parallel with the changing concentration of prothoracic gland hormone (ecdysone). By contrast, they show no clear correlation with juvenile hormone, for the latter declines prior to pupation and is virtually absent during the early phases of adult development (Williams, 1961).

b. Ecdysone vs. the "injury factor"

There seems little reason to doubt that, in one way or another, previously described biochemical and metabolic changes associated with the termination of diapause are directly or indirectly promoted by ecdysone. Yet, strange to say, all of these changes accompanying the action of ecdysone and the initiation of adult development can take place in the absence of ecdysone and without any trace of adult development. It is only necessary to make an injury in the pupal integument.

It is not our present purpose to enter into a detailed discussion of the injury response or the "injury factor" which is apparently released from the wound. These matters have already been subjected to detailed study and review (Shappirio, 1960; Harvey and Williams, 1961; Wyatt, 1963). Suffice it to say that no previously described chemical or metabolic criterion has permitted one to distinguish between a wounded diapausing pupa and a developing moth. Evidently, the biochemical response to ecdysone includes one or more effects which are necessary for development but which cannot be duplicated in response to the injury factor. Therefore, the biochemical difference between injured diapausing pupae and developing adults is a matter of central significance to any comprehensive theory of the mode of action of ecdysone.

The present study has directed attention to what appears to be the first such difference that has been recognized. For most cell types, the prothoracic gland hormone constitutes a potent stimulus for DNA synthesis, whereas the injury factor does not.

c. Ecdysone and DNA synthesis

Among the various tissues here examined, there appear to be at least three categories of response, in terms of DNA synthesis, to development and to injury:

Pupal hemocytes show a specific response to large injuries by enhanced DNA synthesis and by accumulation at the site of injury. This finding is reasonable in view of the active role which hemocytes play in wound healing (Wigglesworth, 1937; Smith and Schneiderman, 1954; Lea and Gilbert, 1961). An increase in circulating blood cells after injury has been previously noted by several investigators (Harvey and Williams, 1961; Lea and Gilbert, 1961), and the results here indicate that this is a true multiplication and not simply a mobilization of sedentary cells. Pupal hypodermal cells in the immediate vicinity of the wound also show a specific response to injury by DNA synthesis in a limited number of cells, as previously observed by Davis and Schneiderman (1960).

Certain tissues, such as thoracic myoblasts, the regenerative cells of the midgut, and the tracheoblasts, exhibit a "metastable" response. They respond most markedly at times of high ecdysone titer, *i.e.*, at the pupal molt and at the beginning of adult development. But they also apparently respond to states of heightened metabolism (*e.g.*, injury) by sporadic synthesis of DNA. Although spermatogonia appear to continue a slow rate of DNA synthesis and cell division throughout diapause, *meiotic* divisions are rarely seen in an uninjured diapausing pupa. Such divisions are frequently seen in sections of testes taken from animals during the pupal molt, at the beginning of adult development, and after large injuries—that is, at times of heightened metabolism. The cysts which show meiosis at pupation and after injury degenerate without forming spermatozoa (Bowers, unpublished observations).

Finally we may note a third category of tissues—the hypodermis (with the exception of the margins of a wound) and the abdominal intersegmental muscles—which seem to synthesize DNA only when ecdysone is being secreted. Thus these tissues appear to respond specifically to the presence of ecdysone. Similar findings have been reported for *Rhodnius* (Wigglesworth, 1963) where ecdysone is necessary for the "activation" of epidermal cells and intersegmental muscles, but

is not necessary for the activation of fat body or hemocytes. In the case of Cecropia, however, the analogy does not extend to fat body, since this tissue was not observed to synthesize DNA until after visible initiation of development.

It seems not unlikely that detailed and quantitative studies of DNA synthesis in injured pupae and in developing adults may lead to a more intimate understanding of the mode of action of ecdysone.

Summary

1. DNA synthesis at stages in the life-cycle of the Cecropia and Cynthia silkworms was examined by radioautographic survey of the incorporation of tritiated thymidine.

2. In sections of pupae previously injected with tritiated thymidine, incorporation of the nucleotide into DNA, as well as cell division, was observed in several tissues as late as one week after pupation.

3. In diapausing pupae, incorporation of thymidine occurred in spermatogonia, hemocytes, a few midgut regenerative cells, and a few cells of the testicular walls. Neither incorporation nor mitotic figures were observed in diapausing epidermal tissues.

4. Large epidermal injury increased DNA synthesis in the blood cells of diapausing pupae and induced incorporation in a few epidermal cells in the immediate vicinity of the wound. Epidermal cells outside the wound periphery did not incorporate thymidine after injury.

5. A generalized incorporation of thymidine was observed in synchrony with the termination of pupal diapause. The first cells to show increased incorporation were the regenerative cells of the midgut, followed by cells of the epidermis, muscles, nerves, tracheoblasts, and, ultimately, the fat body. The incorporation in the epidermis precedes and then accompanies the extensive cell division associated with adult differentiation.

6. Lack of DNA synthesis in the epidermal tissues of injured diapausing pupae appears to be the first biochemical or metabolic criterion so far recognized that permits one to distinguish between an injured pupa and a developing adult.

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